

Classification of common variable immunodeficiencies using flow cytometry and a memory B-cell functionality assay

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Background: The population of patients with common variable immunodeficiency (CVID) comprises a heterogeneous group of patients with different causes of hypogammaglobulinemia predisposing to recurrent infections, higher incidence of autoimmunity, and malignancy. Although memory B cells (memBcs) are key players in humoral defense and their numbers are commonly reduced in these patients, their functionality is not part of any current classification.

Objective: We established and validated a memBc enzyme-linked immunosorbent spot (ELISpot) assay that reveals the capacity of memBcs to develop into antibody-secreting cells and present an idea for a new classification based on this functional capacity.

Methods: The memBc ELISpot assay, combined with flow cytometry, was applied to patients with confirmed CVID in comparison with age-matched healthy control subjects.

Results: *Ex vivo* frequency of IgG-, IgM-, and IgA-secreting plasmablasts was significantly diminished by 27.2-, 2.4-, and 23.3-fold, respectively, compared with that seen in healthy control subjects. Moreover, *in vitro* differentiation of memBcs into antibody-secreting cells was 6.1-, 2.6-, and 3.7-fold significantly reduced for IgG-, IgM-, and IgA-secreting cells, respectively. Proliferation of memBcs correlates inversely to immunoglobulin-secreting capacity, suggesting compensatory hyperproliferation. Furthermore, patients with no serum IgA can still have a detectable IgA ELISpot assay result *in vitro*. Most importantly, the large heterogeneity of memBc function in patients with CVID homogeneously grouped by means of fluorescence-activated cell sorting allowed additional subclassification based on memBc/plasmablast function. **Conclusion:** These data suggest almost normal memBc/immunoglobulin-secreting plasmablast functionality in some patients if sufficient stimulatory signals are delivered, which might open up opportunities for new therapeutic approaches. (J Allergy Clin Immunol 2014;■■■■:■■■■-■■■■.)

Key words: Memory B cell, enzyme-linked immunosorbent spot assay, common variable immunodeficiency subtyping, flow cytometry, antibody-secreting cells

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Common variable immunodeficiency (CVID) is a primary immunodeficiency presenting as a heterogeneous phenotype associated with recurring infections, mainly of the respiratory tract, potentially leading to chronic lung disease, autoimmunity, lymphoid infiltration, enteropathy, and a higher risk of malignancies.¹⁻⁵ With an incidence of about 1:25,000 to 1:50,000 in Western countries, CVID is the most common symptomatic primary immunodeficiency.^{1,6,7} For probable CVID, current diagnostic criteria according to the European Society for Immunodeficiencies are a marked reduction in IgG levels (≥ 2 SDs less than the age mean) and a clear decrease in levels of at least 1 of the isotypes IgA and IgM, absent isohemagglutinins, and/or poor response to vaccines and onset of immunodeficiency at more than 2 years of age when defined causes of hypogammaglobulinemia have been excluded (www.esid.org).^{3,6} Genetic analysis has identified defects in inducible costimulator,⁸ CD19,⁹ CD20,¹⁰ CD21,¹¹ CD81,¹² B cell-activating factor receptor,¹³ and LPS-responsive beige-like anchor¹⁴ in less than 10% of patients with CVID, leaving more than 90% of the cause unknown.^{2,7,15-18} Memory B cells (memBcs) are formed during T cell-dependent antigen encounter and ensure subsequent rapid immune responses with vast amounts of high-affinity antibodies,¹⁹⁻²² and their numbers are often greatly reduced in patients with CVID.^{5,18,23,24}

Abbreviations used

ASC:	Antibody-secreting cell
CVID:	Common variable immunodeficiency
ELISpot:	Enzyme-linked immunosorbent spot
FACS:	Fluorescence-activated cell sorting
HC:	Healthy control
Ig-sPb:	Immunoglobulin-secreting plasmablast
memBc:	Memory B cell
PB:	Pacific Blue
PE:	Phycoerythrin

Many ways to improve the diagnostics of CVID have been proposed over the past decades,^{2,16,17,23,25,26} sometimes without sufficiently reflecting the heterogeneity of the disease,² which still constitutes a major challenge for classification.^{5,17} Currently, serum immunoglobulin levels, particularly representing long-lived plasma cell function, and flow cytometry (fluorescence-activated cell sorting [FACS]), showing numeric B-cell subgroup deficiencies, are the gold standard for CVID diagnostics and classification.^{6,23,26} The most recent scheme, EUROclass, combines the Freiburg²⁶ and Paris²⁷ classifications. Patients are separated into subgroups according to their B-cell counts, then based on memBc counts, and finally based on further B-cell subpopulations, such as activated and transitional B cells, to be correlated with clinical data.^{23,28} Memory B-cell function (ie, the residual capacity to respond to rechallenge with pathogens) has been described^{29–31} but is not part of any commonly used clinical test, even though these cells are a vital part of humoral defense and their numbers are known to be reduced in many patients with CVID.^{5,23} Therefore, we established a well-standardized and validated memBc enzyme-linked immunosorbent spot (ELISpot) assay. The *in vitro* ELISpot assay detects functioning antibody-secreting cells (ASCs)³² attained from reactivated memBcs during stimulation culture,²⁰ allowing us to distinguish between several functional deficits and thereby facilitating a more accurate classification when used in combination with FACS. Additionally, the *ex vivo* memBc ELISpot assay can reveal *in vivo*-activated immunoglobulin-secreting plasmablasts (Ig-sPbs). A more detailed classification might be the basis of a personalized therapy for CVID.^{6,33} Identifying patients with remaining functional memBcs might reveal a group of patients needing less IgG substitution.^{1,34} In addition, almost normal *in vitro* memBc function in the presence of sufficient costimuli might indicate options for new therapeutic approaches to partly replace IgG substitution therapy.

METHODS**Blood samples and clinical data from patients and healthy subjects**

After the immunoglobulin ELISpot assay and flow cytometric panels were established and validated, fresh blood samples from 14 patients with confirmed diagnosis of CVID were compared with samples from 47 healthy control (HC) subjects. CVID was defined based on the criteria of the European Society for Immunodeficiencies.³

This study was approved by the Institutional Review Board of Charité Berlin for HC subjects in October 2010 (EA1/250/10) and for patients in June 2012 (EA2/046/12). All participants provided written informed consent.

One aliquot of each sample was used for flow cytometry and the *ex vivo* ELISpot assay revealing Ig-sPbs, and the second aliquot was tested for

functional capacity to differentiate into ASCs during *in vitro* stimulation (Fig 1).

The HC group (n = 47 adults), consisting of an equal number of male and female subjects in the age groups of 18 to 30, 31 to 40, 41 to 50, 51 to 60, and 61 to 70 years, showed no significant age- or sex-related differences of response (see Fig E1 in this article's [Online Repository](http://www.jaci-online.org) at www.jaci-online.org).

Memory B-cell ELISpot assay before and after stimulation

PBMCs were flow cytometrically analyzed before and after culture under standard incubation settings for 6 days in 6-well plates at 3e6 cells/3 mL complete RPMI with the stimulation cocktail.³⁵ In brief, we used 6 µg/mL of the Toll-like receptor 9 ligand CpG,³⁶ 1:10,000 *Staphylococcus aureus* Cowan activating Toll-like receptor 2, 1 ng/mL pokeweed mitogen with a great effect on B-cell proliferation and T-cell activation,³⁷ and 50 µmol/L β-mercaptoethanol stimulating B- and T-cell help, according to the method of Crotty et al,³⁵ to achieve optimal antigen-unspecific stimulation.^{38–40}

ELISpot assay was performed with standardized 96-well plates commercially coated by our partner, GenID (Straßberg, Germany), to detect total IgG, IgM, and IgA secretion at several concentrations per well (2.5×10^5 *ex vivo* and 1.25×10^4 , 6.5×10^3 , 3.1×10^3 , and 1.5×10^3 *in vitro*). After incubation for 2 hours at standard settings, plates were washed, and biotinylated secondary antibody was applied overnight. Detection was performed with 1 hour of streptavidin, washing, and 3 to 5 minutes of 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt to induce color development. Once the reaction was stopped and the plates dried, they were analyzed with the ELISpot Reader (ImmunoSpot Cellular Technology Ltd, Cleveland, Ohio). The *ex vivo* ELISpot assay from freshly isolated PBMCs detects *in vivo*-activated Ig-sPbs, and the ELISpot assay from *in vitro*-stimulated PBMCs reveals ASCs from reactivated memBcs for the class-switched isotypes IgG and IgA, but we cannot exclude formation of ASCs from both naive B cells and memBcs for IgM.

EUROclass panel FACS staining

Samples were prepared from fresh and cultured PBMCs, as described for the ELISpot assay. Cells were stained with LD-PB (Invitrogen, Carlsbad, Calif) in 100 µL for 20 minutes at 4°C before adding the antibodies for 10 minutes at room temperature. EUROclass panel mix 1 includes anti-CD3–Pacific Blue (PB), anti-CD27–phycoerythrin (PE), anti-IgD–fluorescein isothiocyanate (BD Biosciences, San Jose, Calif), anti-CD14–PB, anti-CD16–PB (Invitrogen), anti-CD19–PE-Cy7, anti-IgM–allophycocyanin (Beckman Coulter, Fullerton, Calif), and mix 2 contains the same antibodies, except for anti-CD21–PE and anti-CD38–fluorescein isothiocyanate (BD Biosciences) replacing anti-CD27 and anti-IgD, respectively. Counting Beads (Invitrogen) were added to a sample of cells from the suspension plated for culture, as well as directly from the culture plate after stimulation, and stained with anti-CD3–PB and anti-CD19–PE-Cy7, as mentioned above.⁴¹ Gating on leukocytes, singlets, and living CD19⁺ cells, we marked naive cells (IgD⁺CD27[−]), marginal zone cells (IgD⁺CD27⁺), memBcs (IgD[−]CD27⁺), and plasmablasts (CD19^{dim}38^{high}), according to the method of Wehr et al.²³

Software and statistics

For flow cytometric analysis, all samples were measured on an LSR II (BD Biosciences) by using FACSDiva (version 6.1.3, BD Biosciences) and FlowJo (version 9.2; TreeStar, Ashland, Ore) software; for ELISpot assay readout, ImmunoCapture (version 6.0) and ImmunoSpot Academic (version 4.17; Cellular Technology, Shaker Heights, Ohio) software was used, and for statistical analysis, GraphPad Prism (version 5.0; GraphPad Software, La Jolla, Calif) software was used. Precision was determined by calculating the coefficient of variation. Mann-Whitney *U* tests were used for comparing the HC and patient groups. Spearman correlation coefficients (*r*) are noted when used. Statistical significance was defined as a 2-sided *P* value of less than .05.

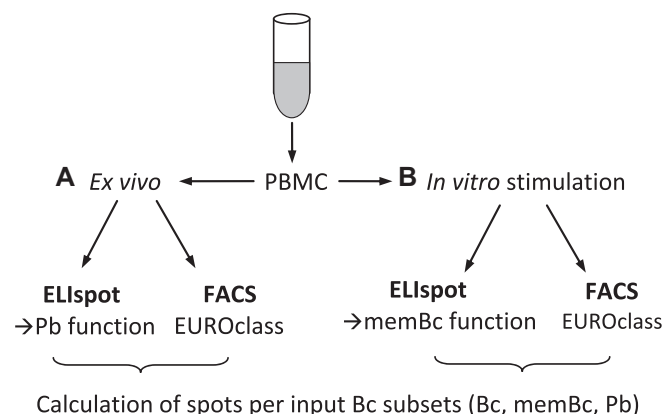


FIG 1. Study scheme. After drawing heparinized peripheral blood and the density gradient separation of PBMCs, cells were divided into 2 samples, one for *ex vivo* analysis of B-cell counts and Ig-sPBs which detect *in vivo* activation (A) and one for *in vitro* stimulation of B cells for 6 days to measure the functional capacity of B cells differentiating into ASCs during adequate helper/stimulatory signals (B). Bc, B cells; Pb, plasmablasts.

RESULTS

Precision of the memBc ELISpot assay

To determine the precision of our method, we performed quality control experiments and calculated the coefficient of variation (CV) as follows:

$$CV [\%] = (SD/\text{mean}) * 100.$$

Analyzing 5 separate blood samples from the same healthy donor on the same day revealed an intra-assay variance of 16%, 11%, and 26% for IgG, IgM, and IgA, respectively. The interassay variance test, using 5 replications from the same healthy donor on 5 consecutive days, showed a precision of 45%, 46%, and 47% for IgG, IgM, and IgA, respectively (see Fig E2 in this article's Online Repository at www.jacionline.org). Also, we analyzed 4 patients with CVID in 5 repetitions to check the feasibility of reproducible classification. In fact, the intra-assay and interassay CVs were similar to those of HC subjects or even a little bit higher because of lower frequencies. Nevertheless, on the basis of our selected categorization (>25% of mean in HC subjects = normal responder, 10% to 25% of mean of HC subjects = low responder, and <10% of mean of HC subjects = nonresponder), we stratified almost all 20 samples (5 each of the 4 patients) into the same category. Only 1 of 20 samples was misclassified into 1 subclass higher.

Subgroups of CVID according to flow cytometric memBc numbers

Subjects' characteristics are shown in Table I. All patients had greater than 1% B cells of total lymphocytes. By using functional testing with the memBc ELISpot assay, the patients with CVID were stratified according to our new classification system consisting of 3 groups: I, almost normal functionality (>25% of HC mean); II, reduced but detectable functionality (10% to 25% of HC mean); and III, almost no functionality (<10% of HC mean). This new system was then compared with the Freiburg classification, as well as clinical data (Table II). Patients were also divided into 2 groups according to the Freiburg classification,²⁶ which we used because it was shown to be more predictive

TABLE I. Subjects' characteristics

	HC subjects	Patients with CVID*
No.	50	14
Sex (female/male)	25/25	6/8
Age (y),† mean ± SD	44 ± 15.4	46 ± 14.2
Age at diagnosis (y), mean ± SD	—	30 ± 15.2
IgA + IgG deficiency	0	2
IgM + IgG deficiency	0	1
IgA + IgM + IgG deficiency	0	11
Substitution subcutaneous/intravenous/none	0	11/1/2

*Freiburg classification of CVID: FI group, n = 5; FII group, n = 9.

†Mann-Whitney U test: P = 1.0 (not significant).

regarding the capacity to produce immunoglobulin *in vitro* than the Paris²⁴ and the frequently used EUROclass²³ schemes. Patients in Freiburg group FI (n = 5 patients) have less than 0.4% and those in Freiburg group FII (n = 9 patients) have greater than 0.4% CD27⁺IgM⁺IgD⁺ memBcs of total lymphocytes.²⁶

Diminished *ex vivo* frequency of Ig-sPBs/ASCs

Serum immunoglobulin is mainly derived from long-lived plasma cells in the bone marrow. Activated B cells differentiate into Ig-sPBs in the lymph nodes, spleen, or both and then circulate in peripheral blood and home to the bone marrow or inflammatory sites. We addressed the question of whether serum immunoglobulin levels, a key diagnostic parameter, are related to the immunoglobulin-secreting capacity of plasmablasts in peripheral blood by performing *ex vivo* ELISpot assays to quantify them. Fig 2 shows a significant 7.3- and 123-fold reduction of total Ig-sPB numbers for the FII and FI groups, respectively, compared with those seen in HC subjects when calculated per 10,000 total CD19⁺ cells (including CD19^{dim} plasmablasts). Most importantly, both groups of patients showed broad heterogeneity, with some showing almost normal frequencies (particularly for IgG plus IgM), and in other patients Ig-sPBs were barely detectable at all (Fig 2). IgA-sPBs were most strongly affected.

Diminished *in vitro* differentiation of memBcs into ASCs

Next we studied whether memBcs are able to differentiate into ASCs *in vitro* if adequate stimuli are sufficiently delivered within the culture period of 6 days. Fig 3, A, shows the significantly diminished number of ASCs after *in vitro* expansion of FII group patients for all immunoglobulin classes, which were even more deteriorated within FI group patients. However, broad heterogeneity was seen again among the patients, particularly those of the FII group, ranging from almost normal responses to values diminished by several log levels compared with those seen in HC subjects (Fig 3).

Serum IgG, but no other immunoglobulin classes, correlate with respective ELISpot assay results

To address the question of whether the extent of immunoglobulin deficiency was related to residual *ex vivo* or *in vitro* plasmablast/memBc function, we compared ELISpot assay data with the residual serum immunoglobulin levels before IgG substitution (ie, at the time of diagnosis; Fig 4). Results of the

TABLE II. New classification compared with Freiburg classification and clinical data

Patients	Freiburg classification	Classification based on <i>in vitro</i> functionality (total CD19 B cells)			Classification based on <i>in vitro</i> functionality (memCD19 B cells)		Sinusitis	Pneumonia	Autoimmunity	Splenomegaly	Lymphadenopathy
		Type	IgG	IgM	IgA	IgG	IgA				
P2	Ia		*	‡	*	*	*	+	—	—	—
P12	Ib		*	*	*	*	*	+	+	+	—
P3	Ib		*	‡	*	‡	*	+	—	—	+
P1	Ib		*	‡	*	*	‡	+	+	—	+
P13	Ib		*	‡	*	‡	*	—	—	+	+
P4	II		*	*	*	*	*	+	—	+	—
P11	II		*	‡	*	*	*	—	—	+	—
P9	II		*	‡	*	‡	*	—	—	+	—
P5	II		*	‡	*	‡	*	+	—	—	—
P15	II		‡	‡	*	‡	*	+	+	+	—
P14	II		‡	‡	‡	‡	‡	+	+	—	+
P7	II		*	‡	‡	*	‡	+	+	—	—
P8	II		‡	‡	‡	‡	‡	—	—	—	—
P10	II		‡	‡	‡	‡	‡	—	—	—	—

Freiburg classification counts CD19 memBcs. Patient order: primarily by Freiburg classification and within groups FIa, FIb, and FII by residual B-cell function from low to high. Patient 6 was excluded because of B-cell counts of less than 1%, which is a criterion for Freiburg classification. Functional test (total CD19 B cells plus CD19 memCD19Bcs): *nonresponder (<10% of HC subject mean), †low responder (10% to 25% of HC subject mean), ‡almost normal (>25% of HC subject mean).

+, Present; —, not present; 0, splenectomy.

ex vivo ELISpot assay detecting Ig-sPbs showed no relation to pre-IgG substitution serum immunoglobulin levels (data not shown). By contrast, patients' pretreatment serum IgG levels correlated significantly with the IgG ELISpot assay enumerating ASCs after *in vitro* stimulation ($r = 0.7477$, $P = .0172$; Fig 4, B); however, no correlation was observed for the other immunoglobulin classes (Fig 4, C and D). This proposes a relation between the residual functionality of IgG⁺ memBcs and serum IgG levels in patients. For HC subjects, no correlation could be found for either *ex vivo* or *in vitro* ELISpot assays, confirming that serum immunoglobulin levels are almost completely related to long-lived plasma cells in the bone marrow (see Fig E3 in this article's [Online Repository](#) at www.jacionline.org).²²

Enhanced memBc proliferation rates correlate with low ASC frequencies

The efficiency of *in vitro* culture to generate ASCs relies on the ability to activate and induce memBc proliferation. To determine whether diminished memBc expansion could be the reason for reduced ASC frequencies, we calculated the quotient of absolute B-cell counts measured by using FACS before (input) and after (output) *in vitro* stimulation culture. Surprisingly, HC subjects and FII group patients had similar B-cell expansion rates, whereas FI group patients had significantly enhanced expansion compared with both HC subjects ($P = .023$) and FII group patients ($P = .019$; Fig 5, A). This was even more prominent when only input/output memBcs were calculated as a percentage of absolute B cells, showing a 7.5-fold ($P = .031$) and 16.3-fold ($P = .002$) increase of memBc proliferation for FI and FII group patients compared with HC subjects, respectively (Fig 5, B). Again, proliferation rates of FII group patients showed a broad range from almost normal to strongly enhanced levels (minimum of 2.8-fold to maximum of 93.6-fold increase; mean, 24.5-fold). However, the memBc proliferation rate correlates inversely ($r = -0.64$, $P = .0138$) to ELISpot assay results after stimulation (Fig 5, C).

Dissection of immunoglobulin class responses in patients versus HC subjects: Switched memBc/plasmablast response is strongly diminished

Next, we analyzed Ig-sPbs/ASCs in the PBMC fraction *ex vivo* and after *in vitro* expansion. *Ex vivo* ELISpot assay results of patients with CVID are greatly reduced compared with those of HC subjects ($P < .0001$), as seen in Table III. Although IgA-sPbs dominate the response in HC subjects, in patients most of the Ig-sPbs secrete IgM (Fig 6 and Table I).

Stimulation of memBcs from HC subjects results in dominant IgM (76%) responses, which are even stronger in patients (IgM, 89%) but show about 2.4 times less IgG compared with that seen in HC subjects after culture. To analyze the cellular source of immunoglobulin secretion after *in vitro* stimulation, we sorted the cells and used either total B cells or memBc-depleted B cells for the ELISpot assay. The results revealed that the *in vitro* IgM spots mainly reflect the response of naive B cells activated during this stimulation. In contrast, IgG/IgA responses are almost completely dependent on memBcs (data not shown).

Dissection of functional response in relation to input cell subtypes: Functional heterogeneity among patient groups

Next, we investigated whether the diminished ELISpot assay response in most patients is related to functional or numeric deficiency of B cells. Fig 3 shows the number of ASCs measured by using the ELISpot assay adjusted for the number of input B-cell subsets counted by using flow cytometry. Even after adjustment for input B cells or input memBcs (reasonable only for IgG/IgA response because of their memBc dependency), patients' responses are significantly reduced compared with those of HC subjects. The broad heterogeneity of IgG/IgA responses, particularly among group FII patients, is also detectable after correction of input memBc counts (Fig 3, Bi and Biii), reaching more than 2 log levels from almost normal to strongly diminished

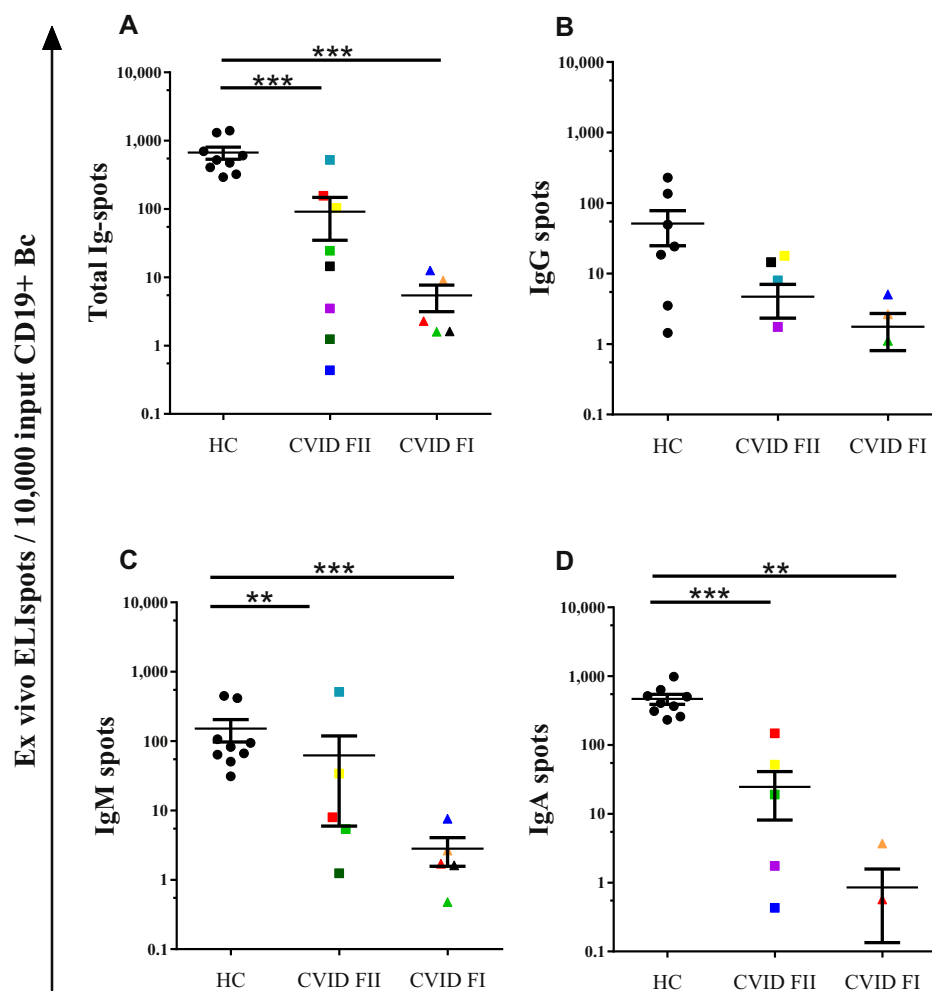


FIG 2. Diminished Ig-sPb function in patients with CVID detected by using the *ex vivo* ELISpot assay. The y-axis shows total immunoglobulin (A), IgG (B), IgM (C), and IgA (D) ELISpot assay results *ex vivo* calculated per 10,000 input CD19⁺ and CD19^{dim} B cells, thereby including plasmablasts in flow cytometry. HC subjects and patients with CVID classified by using the Freiburg scheme are grouped on the x-axis. Mann-Whitney U test: ****P* < .001 and ***P* < .01. Whiskers depict SEMs. Bc, B cells.

responses. In addition, the data also reveal a deficiency of CD38⁺ (pre)plasmablasts secreting IgG and IgA in FII group patients (Fig 3, Ci-Ciii). In the FI group, secretion of all immunoglobulin classes is diminished by CD38⁺ (pre)plasmablasts. Again, a broad heterogeneity among FII group patients is observed.

Finally, the data show that even B cells of patients with almost undetectable serum IgG, IgM, and IgA can differentiate into ASCs if adequate signals are delivered *in vitro*. For example, 5 of 15 patients from both groups FI and FII (FII, c, e, and h; FI, b and c) with almost no serum IgA expressed significant IgA responses *in vitro*. The PBMC data from which we calculated Fig 3 can be seen in Fig E4 in this article's [Online Repository](http://www.jacionline.org) at www.jacionline.org.

T-cell cytokine effect on B-cell development in culture

To assess T-cell activity under the conditions we used for B-cell differentiation into antibody-producing cells, we analyzed cytokine levels in culture supernatants by means of multiparameter cytometric bead array flow cytometric analysis,

showing strongly reduced levels of IL-2 and marginally reduced levels of TNF but normal levels of IL-4, IL-5, IL-10, and IFN- γ (see Fig E5 in this article's [Online Repository](http://www.jacionline.org) at www.jacionline.org).

DISCUSSION

The pathogenesis of CVID is complex, and despite revelation of many mutations, the molecular genetics are known in a minority of patients only. Consequently, defects can be found at different levels during B-cell maturation to Ig-sPbs/ASCs, and the clinical picture is just as heterogeneous. The most commonly used CVID categorization is based on flow cytometric memBc counting. Adequate monitoring of patients with CVID is not easy because of the broad range of defects and symptoms.⁶ Chapel et al² suggest the diagnostic criteria for CVID need revision because serum IgG levels showed such a wide range among patients. In line with this notion, our study demonstrates that phenotypic classification according to memBc numbers reflects only a small part of the broad functional heterogeneity among patients with CVID.

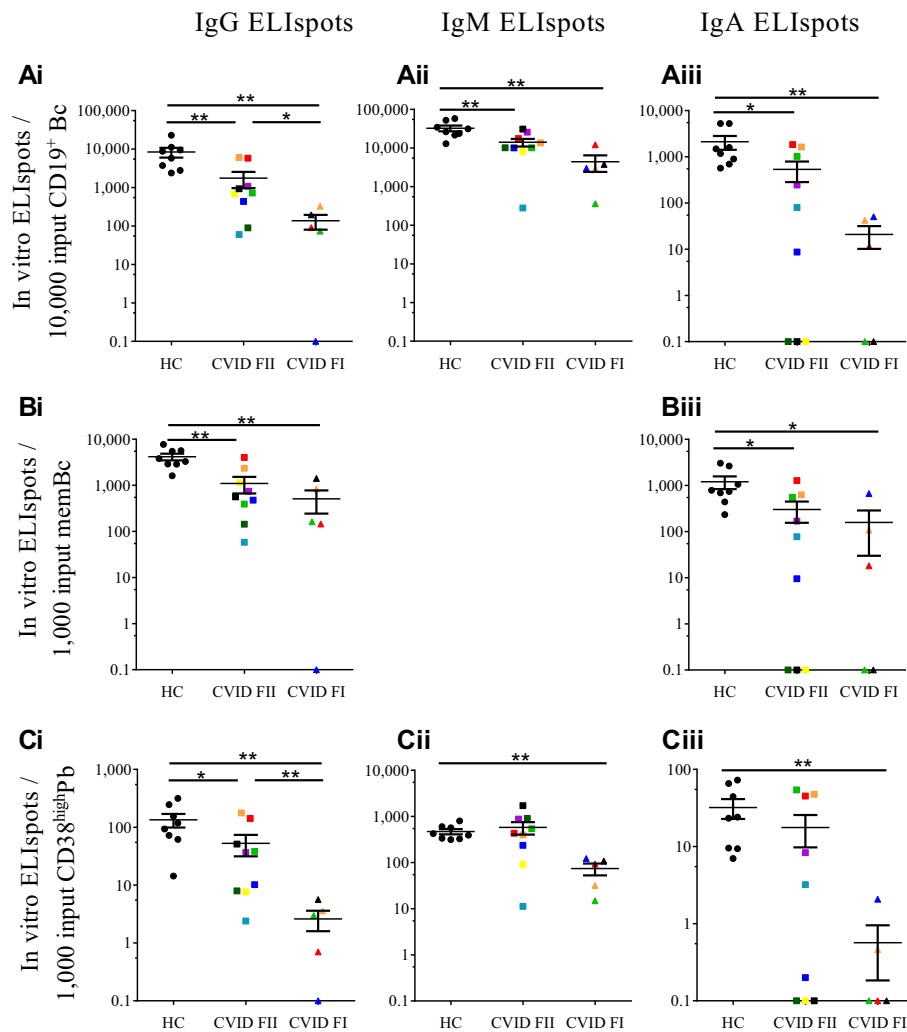


FIG 3. Great heterogeneity among functional responses of patients with CVID are deemed homogeneous when grouped by using flow cytometry, particularly in group FI. Combined analysis of IgG, IgM, and IgA ELISpot assays *in vitro* per input B-cell subset measured by using flow cytometry in HC subjects and patients with CVID classified by using the Freiburg scheme. *In vitro* ELISpot assays (number of ASCs, y-axis) resulting mainly from reactivated memBcs calculated per absolute input cell counts before stimulation of 10,000 CD19⁺ B cells (**Ai-Aiii**), 1,000 CD19⁺27⁺IgD[−] memBcs (**Bi-Biii**), and 1,000 CD19^{dim}38^{high} plasmablasts (**Ci-Ciii**), respectively. There is no image Bii because the IgM response is mainly derived from naive B cells activated during stimulation, and calculation per memBc is not reasonable. The columns represent IgG (*i*), IgM (*ii*), and IgA (*iii*) ELISpot assays. Freiburg classification: FI group patients have less than 0.4% (triangles) and FII group patients have greater than 0.4% (squares) CD27⁺IgM[−]IgD[−] memBcs of total lymphocytes. Mann-Whitney *U* test: ***P* < .01 and **P* < .05. Whiskers depict SEMs.

First of all, numbers of *ex vivo* Ig-sPbs are reduced for all immunoglobulin classes in patients with CVID from both groups, with broad variability over 4 log levels ranging from normal to almost undetectable levels. This suggests normal *in vivo* differentiation into circulating, probably short-lived Ig-sPbs in some patients with CVID independent of the Freiburg classification.

Second, the capacity of memBcs to differentiate into ASCs after optimal *in vitro* stimulation is diminished, again with great heterogeneity among the patients, particularly within the FII group. This suggests that flow cytometry underestimates the deficiency complexity. In contrast to *ex vivo* plasmablast counts, there was no complete nonresponder for IgG and IgM, and only very few patients had no IgA response (Fig 3, Ai-Aiii). These data reveal that sufficient delivery of helper/stimulatory signals

triggers full differentiation into ASCs in many patients. This is even more visible if ELISpot assay data are adjusted for input memBcs (Fig 3, Ci-Ciii), which reduce the differences among patients and HC subjects with a broad range from almost normal to 100 times less response for IgG and IgA. Interestingly, the IgM response is almost normal in all patients. This could be explained by the fact that the IgM response after stimulation is mostly due to naive B-cell activation.

This suggests that the deficiency is caused in at least some patients by inadequate *in vivo* delivery of signals to the memBc compartment to differentiate into ASCs rather than intrinsic B-cell defects.

Although serum immunoglobulin in HC subjects is derived mostly from long-lived plasma cells in bone marrow niches, we observed some correlation between the frequency of IgG ASCs

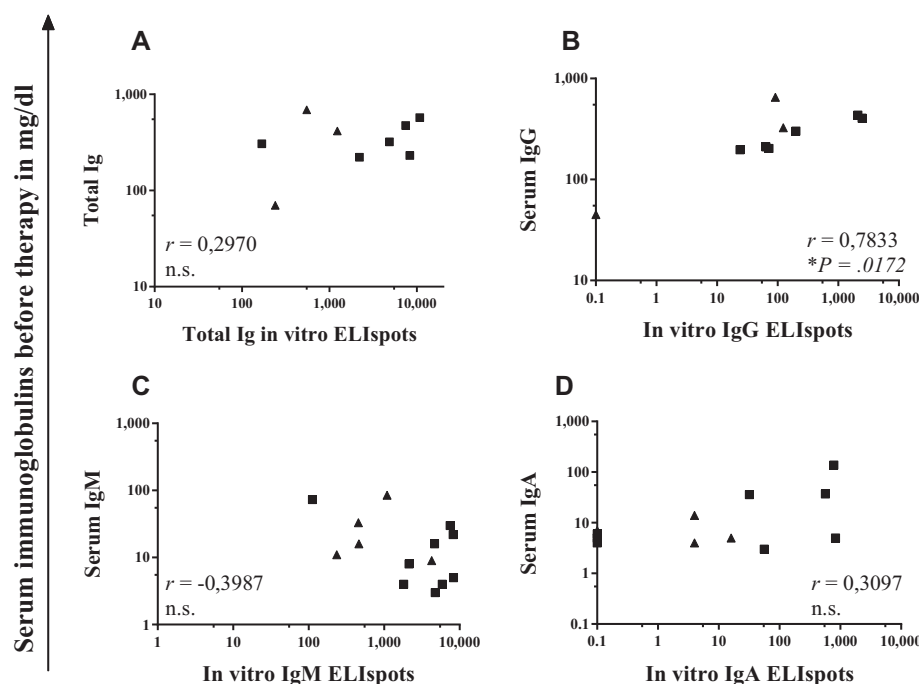


FIG 4. Pretreatment serum IgG level at the time of CVID diagnosis correlates with *in vitro* IgG ELISpot assay results. Serum immunoglobulin levels before substitution (*y*-axis) in correlation with their corresponding ELISpot assay results (*x*-axis) for total immunoglobulin (**A**), IgG (**B**), IgM (**C**), and IgA (**D**) are shown. Freiburg classification by memBcs: FI group patients have less than 0.4% (triangles) and FII group patients have greater than 0.4% (squares) CD27⁺IgM⁺IgD⁺ memBcs of lymphocytes. Serum IgG correlates to IgG ELISpot assay results. Spearman correlation: $r = 0.7833$, $P = .0172$. *n.s.*, Not significant.

after *in vitro* stimulation and pre-IgG substitution IgG levels of patients with CVID on diagnosis (Fig 4, B). These data suggest impaired turnover from memBcs into long-lived IgG-secreting plasma cells, resulting in a decrease in serum IgG levels. This was not observed for other immunoglobulin classes.

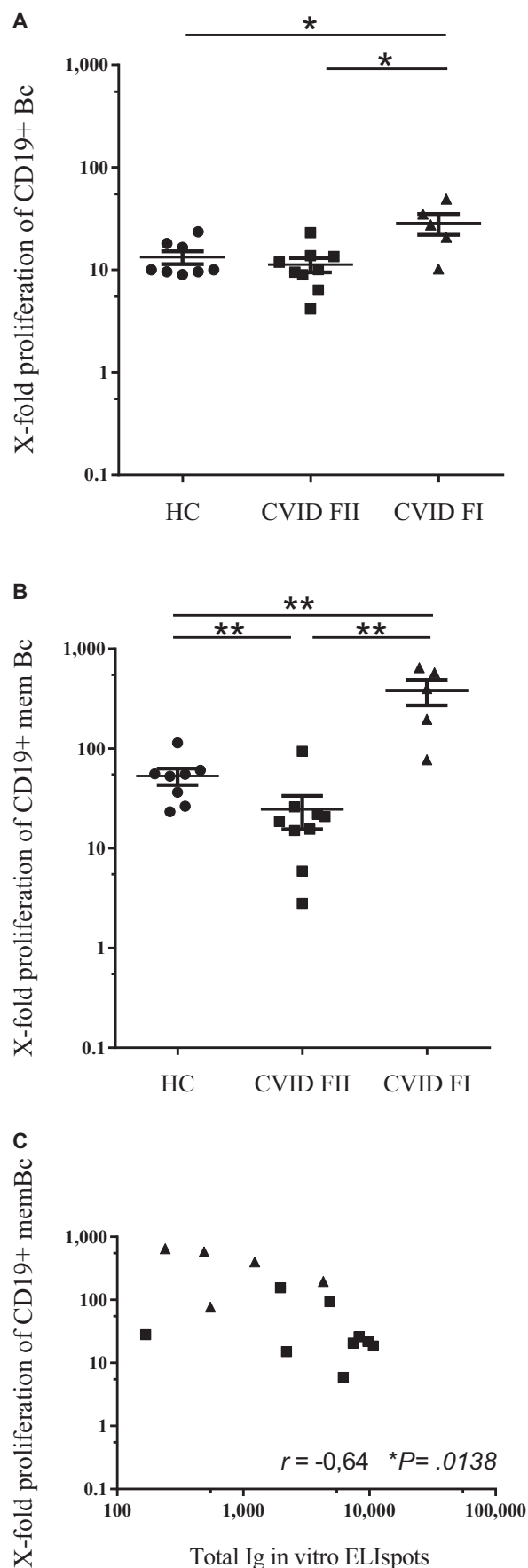
Furthermore, we showed that despite differentiation of memBcs into CD38^{bright} cells (a key marker for plasmablasts) in most patients, the number of ASCs per 1000 input CD38^{bright} cells are reduced in many patients, particularly in the FI group (all immunoglobulin classes) but also in the FII group (reduced IgG and IgA but normal IgM levels). However, the majority of FII group patients had normal responses per input CD38^{bright} cells (Fig 3, Ci-Ciii). These data reveal another roadblock in B-cell differentiation into ASCs in patients with CVID. Regarding the association of functional differences with the clinical picture, the number of patients studied is too small to allow convincing conclusions. However, there are some interesting trends, such as the following (Table II):

- Four of 14 patients expressed lymphadenopathy, all of them classified as being in group FI.
- Splenomegaly was observed in 3 of 13 (1 status post-splenectomy) patients without a link to Freiburg classification (1 in class I and 2 in class II), but all of them had decreased/absent IgG/IgA responses *in vitro*.
- Nine of 14 patients had a recent history of pneumonia, severe sinusitis, or both; 4 of 5 belonged to Freiburg class I. Interestingly, the only patient in Freiburg class I with a normal IgG/IgM response, if calculated per memBcs, was the one without infection (but with idiopathic thrombocytopenic purpura). Absence of IgG response was related to the risk of infection.

- Two of 14 patients, both in Freiburg class II, expressed normal IgG, IgA, and IgM levels *in vitro*. These are the only 2 patients who experienced neither severe infections nor autoimmunity, splenomegaly, or lymphadenopathy, demonstrating their favorable course.
- Three of 9 patients in Freiburg class II had low or almost absent IgG and IgA levels *in vitro*. All of them had both severe infections and autoimmunity.
- Nine of 14 patients had an almost absent IgA level *in vitro*, and 6 of them had autoimmunity. By contrast, none of the 5 patients had almost normal or only decreased IgA levels *in vitro*. It is worth noting that all patients with autoimmunity had insufficient IgA responses, even after full delivery of stimulatory factors *in vitro*, demonstrating an intrinsic IgA-producing deficiency associated with high risk of autoimmunity. By contrast, an almost normal *in vitro* IgA level suggests a favorable course regarding autoimmunity. This might change the screening strategy for autoimmunity of the respective high-risk patients.

Our culture system induces differentiation of memBcs into IgG-, IgM-, or IgA-secreting cells, as well as a clear naive B-cell response for IgM. Plasmablasts are generally defined as CD19^{dim}CD38^{high} cells, which in freshly isolated blood account for less than 3% of total lymphocytes in HC subjects.^{28,42} We suspect that flow cytometric gating on CD19^{dim}CD38^{high} cells does not include all IgA spot-forming cells which are known to be a heterogeneous group.⁴³

A recent study has shown good correlation between the frequency of peripheral CD27⁺IgM⁺IgD⁺ switched memBcs and IgG synthesis *in vitro* measured by using ELISA after 8 days of stimulation with *Staphylococcus aureus* Cowan and



IL-2 for patients with CVID.²⁶ No correlation was found between IgM production *in vitro* and IgM-secreting memBcs, which is in accordance with our findings (Fig 4).

Our observations that memBcs have clearly higher proliferation rates than total B cells (Fig 5, A and B) are in accordance with those of Tangye et al,²¹ who describe more proliferation of the splenic CD19⁺CD27⁺ memBc subset than of CD19⁺CD27⁻ naive B-cells measured by using carboxyfluorescein succinimidyl ester staining.

Similar to our findings of an inverse relation between proliferation of memBcs and differentiation into ASCs after *in vitro* stimulation (Fig 5, C), Driessen et al¹⁷ describe compensating B-cell/T-cell hyperproliferation in patients with reduced cell function. They also present a profound classification into 5 immunologically homogeneous pathophysiologic patterns by using a combination of flow cytometry, analysis of somatic hypermutations, and B-cell replication history. On the other hand, the documented hyperproliferation could also be a biological consequence of a failure of differentiation, as described by Golubev.⁴⁴

CVID has a very heterogeneous clinical severity, and therefore it is not surprising that patients need different amounts of immunoglobulin substitution to reach age-related serum immunoglobulin levels within the normal range^{1,34,45} or to control infections.^{45,46} Optimized therapy can be achieved by identifying patients with more residual functional memBcs and Ig-sPbs/ASCs, who are possible candidates for reduced IgG substitution. Currently, these patients' missing reactions to vaccination indicate impaired memBc function, but the memBc ELISpot assay can quantify this more precisely to aid therapeutic decision making. Because about 85% of patients with CVID receive expensive substitution therapy,⁷ identifying patients requiring lower doses would result in substantially reduced therapeutic costs, as well as increased quality of life. This is especially interesting for the case of pediatric CVID, in which shorter infusion times make an even greater difference in quality of life. Because CVID shows comparable phenotypes in children and adults, this diagnostic assay could be implemented for all ages.³³

Adverse reactions are rare but still occur after infusion.³⁴ Anaphylaxis caused by anti-IgA antibodies in completely IgA-deficient patients is sometimes hard to detect because anti-IgA antibodies are not always detectable in the blood.⁴⁷

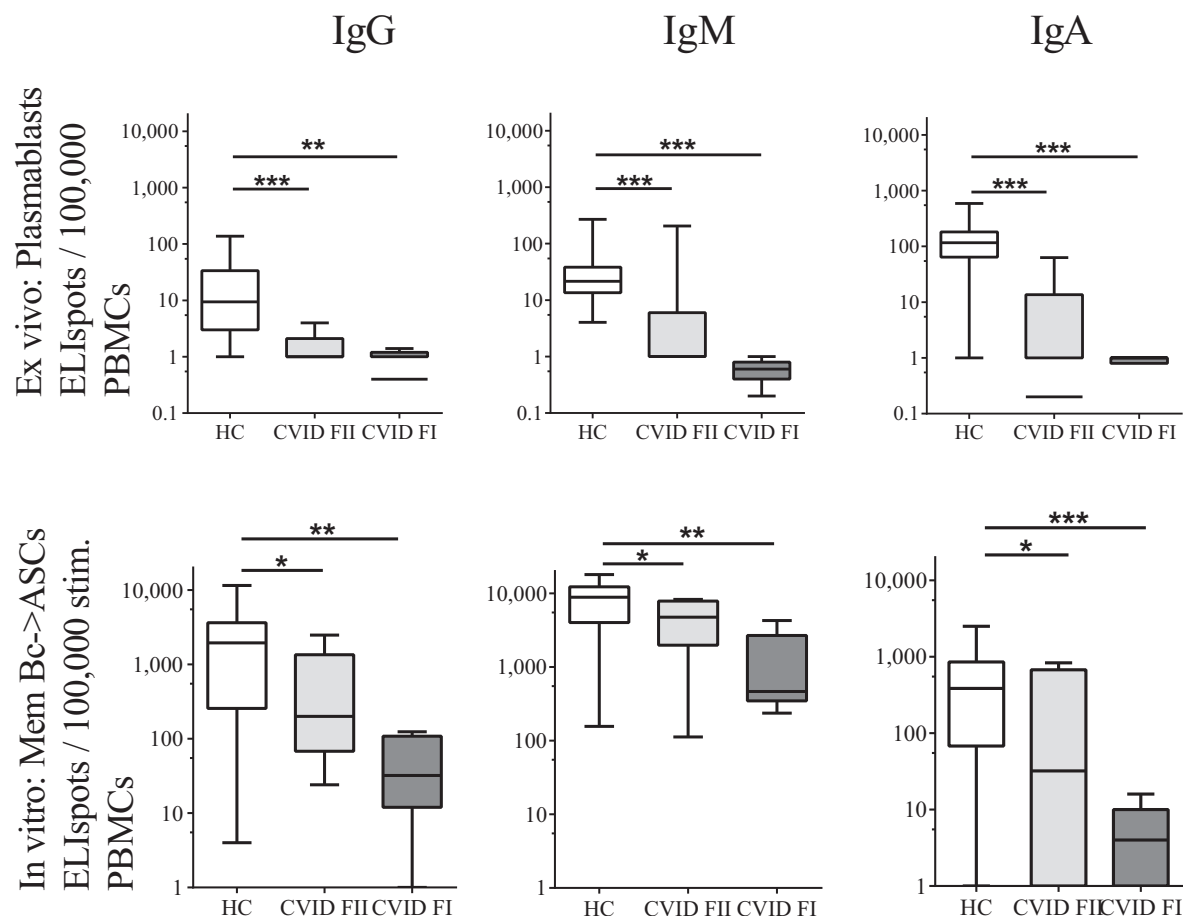
A further possible application might lie in distinguishing transient hypogammaglobulinemia in small children from a persistent disorder, such as CVID.^{1,48}

We observed strongly reduced levels of IL-2 and marginally reduced levels of TNF but normal levels of IL-4, IL-5, IL-10, and IFN- γ in cytometric bead array analysis of culture supernatants (see Fig E5). Likewise, Agarwal et al⁴⁹ also detected reduced IL-2 and TNF- α levels in supernatants from patients with CVID. In contrast to our data, they also observed reduced IFN- γ and IL-10 levels. However, they only checked

FIG 5. Enhanced proliferation rate of memBcs in patients after *in vitro* stimulation correlates with poor differentiation capacity into ASCs. Freiburg classification: FI group patients have less than 0.4% (triangles) and FII group patients have greater than 0.4% (squares) CD27⁺IgM⁺IgD⁻ memBcs of lymphocytes. The y-axis shows X-fold proliferation of respective cell types in flow cytometry. CD19⁺ B cells (A), memBcs (B), memBc proliferation and total ELISpot assay results (C) *in vitro* are shown. Spearman correlation coefficient: $r = -0.64$, $P = .0138$. $*P \leq .0231$ and $**P \leq .0055$.

TABLE III. Comparison of *ex vivo* and *in vitro* immunoglobulin classes in HC subjects and patients with CVID measured by using the memBc ELISpot assay

	IgG	IgM	IgA
X-fold reduction of <i>ex vivo</i> ELISpot assay results in patients with CVID compared with HC subjects	27.2	2.4	23.3
Distribution of <i>ex vivo</i> Ig-sPbs in HC subjects seen in ELISpot assays (%)	69	17	14
Distribution of <i>ex vivo</i> Ig-sPbs in patients with CVID seen in ELISpot assays (%)	43	33	24
X-fold reduction of <i>in vitro</i> ELISpot assay results in patients with CVID compared with HC subjects	6.1	2.6	3.7
Distribution of <i>in vitro</i> Ig-sPbs in HC subjects seen in ELISpot assays (%)	19	76	5
Distribution of <i>in vitro</i> Ig-sPbs in patients with CVID seen in ELISpot assays (%)	8	89	3

**FIG 6.** Functional defects of *in vivo*- and *in vitro*-activated Ig-sPbs/ASCs in patients with CVID compared with HC subjects affects all immunoglobulin classes. The y-axis represents ELISpot assay results per 100,000 PBMCs before (top row) and after (bottom row) stimulation. Patients are grouped by Freiburg classification: FI group patients have less than 0.4% (n = 9) and FII group patients have greater than 0.4% CD27⁺IgM⁺IgD⁺ memBcs of total lymphocytes (n = 5). Box plots are shown with minimum/maximum whiskers. Mann-Whitney U test: *P < .035, **P ≤ .0075, and ***P < .0004.

memory T-cell response by analyzing results after 48 hours of culture, whereas we used different culture conditions, also allowing a naive T-cell response. Of course, this is only a small part of the T-cell effect, which is why T-cell analysis is of future importance.

In conclusion, this study should show where further research is needed to determine whether more functional characterization of patients with CVID by using the memBc ELISpot assay improves current therapy with immunoglobulin substitution and opens up opportunities for more personalized therapy. This might be

especially interesting for patients without serum IgA levels but with existing class-switched IgA memBcs after stimulation. This could be due to missing stimuli *in vivo* given to fully functional cells in the *in vitro* stimulation culture, indicating that some patients have the potential to regain IgA responses when treated with the appropriate stimulation. Because IgA cannot be substituted, these patients would probably show benefit from a new approach of *in vivo* stimulation reducing mucosa-associated infections.⁴⁷ IL-7 can support survival and differentiation of B cells.^{47,48} IL-21 restores immunoglobulin production

ex vivo in patients with CVID and IgA deficiency.⁵⁰ IL-21 also induces class-switching,²² making this a candidate for treating not only patients with existing memBcs but also low class-switched subsets, as well as patients with low memBc counts. This is because treatment with IL-21 also induces a CD27^{high}IgD⁺ (CD38^{low}) population which is in most patients recruited from naive cells.⁵⁰ Avery et al¹⁹ showed IL-21-induced isotype switching to approximately normal IgG levels independent of decreased memBc counts, making this quite interesting for immunodeficient patients. These examples illustrate putative therapeutic strategies in patients with CVID with sufficient *in vitro* memBc differentiation potency to minimize immunoglobulin substitution. Further multicenter studies are necessary to validate the advantage of functional characterization for patient classification.

Further research is needed in terms of prospective studies using the memBc ELISpot assay as a diagnostic tool to test the prognostic value of the test regarding clinical development and risk of long-term damage.

We thank all the participating patients, volunteers, and nurses of the outpatient clinic for immunodeficiencies. We thank Professor Salama (Institute for Transfusion Medicine, Charité) for fruitful discussions. Finally, we thank our cooperation partner, GenID GmbH, for supplying ELISpot assay reagents and methodical discussions, especially Ms Preyer.

Key messages

- Assessment of memory B-cell functionality by using the ELISpot assay in serum immunoglobulin-deficient patients might allow individualized therapy development.
- Combination of the novel memory B-cell ELISpot assay with flow cytometry allows further subgrouping based on functional deficits in patients with CVID.

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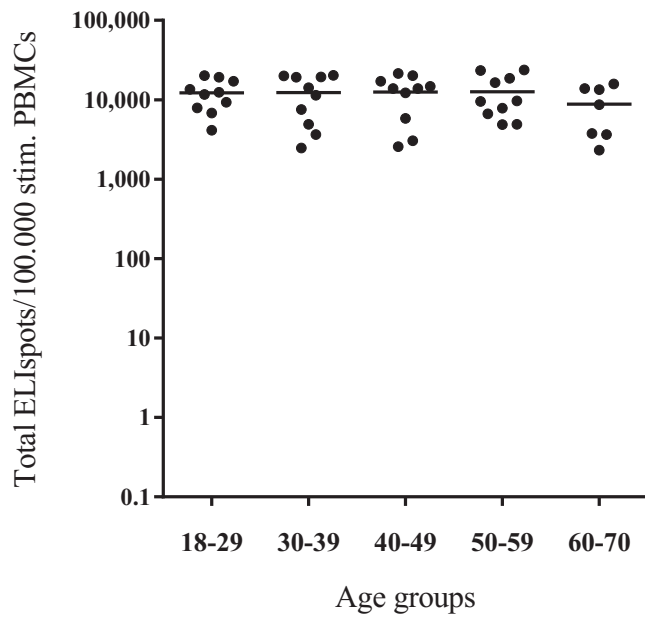


FIG E1. No effect of age on total immunoglobulin ELISpot assay results in adults. Total ELISpot assay result per 100,000 stimulated PBMCs are depicted on the *y-axis* and classified by age group on the *x-axis*. Subjects older than 70 years of age were excluded because of significantly reduced ASC counts (by a factor of 100).

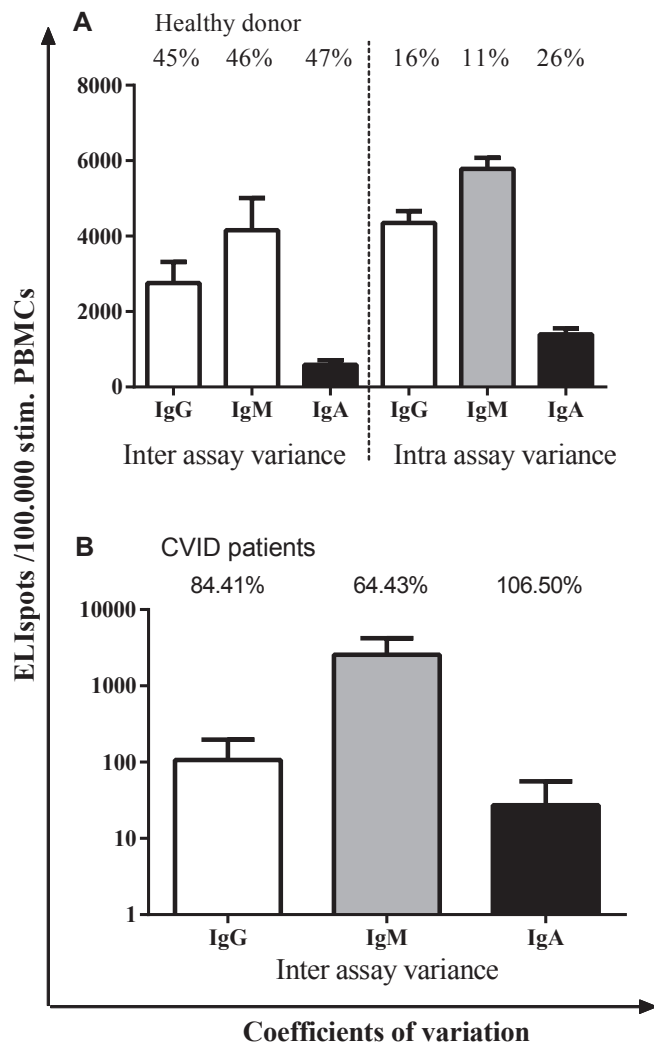


FIG E2. The precision of memBc ELISpot assays is shown by intra-assay and interassay variance tests to be slightly less for patients. The coefficient of variation (CV) is calculated as follows: $(CV [\%] = (s/x) * 100)$, where s is the SD and x is the mean. This shows a test's precision. **A**, Analysis of 5 individual blood samples of 1 healthy donor revealed an intra-assay variance of 16%, 11%, and 26% for IgG, IgM, and IgA, respectively. Interassay variance on 5 consecutive days showed a CV of 45%, 46%, and 47% for IgG, IgM, and IgA, respectively. **B**, Analysis of 5 individual blood samples from 4 patients with CVID showed an interassay variance of 84.41%, 64.34%, and 106.5% for IgG, IgM, and IgA, respectively.

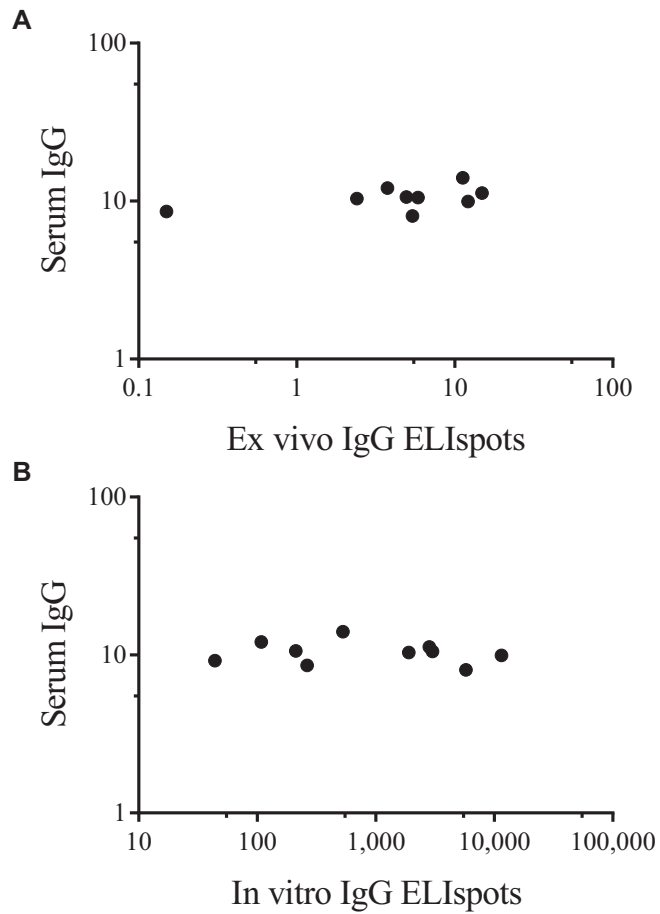


FIG E3. No correlation between serum IgG levels and ELISpot assay results in HC subjects. Total ELISpot assay results per 100,000 stimulated PBMCs are depicted on the *x-axis*, and serum IgG levels are depicted on the *y-axis*. Samples of 10 randomly picked HC subjects were correlated by using the Spearman correlation coefficient: **A**, $r = -0.2364$; **B**, $r = 0.4182$.

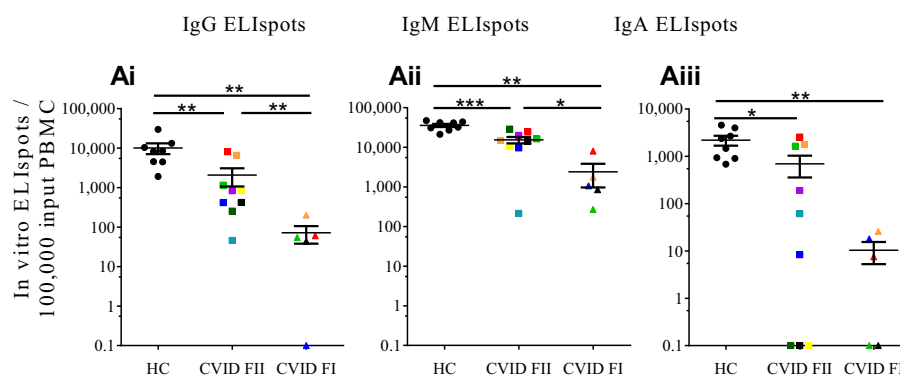


FIG E4. Original data of calculated analysis shown in Fig 6. Combined analysis of IgG, IgM, and IgA ELISpot assay results *in vitro* per input 100,000 PBMCs measured by means of flow cytometry in HC subjects and patients with CVID classified by using the Freiburg scheme. *In vitro* ELISpot assay results (number of ASCs, *y-axis*) mainly from reactivated memBcs calculated per absolute input cell counts before stimulation are shown. The columns represent IgG (Ai), IgM (Aii), and IgA (Aiii) ELISpot assay results. Freiburg classification: FI group patients have less than 0.4% (triangles) and FII group patients have greater than 0.4% (squares) CD27⁺IgM⁺IgD⁻ memBcs of total lymphocytes. Mann-Whitney *U* test: ****P* < .001, ***P* < .01, and **P* < .05. Whiskers depict SEMs.

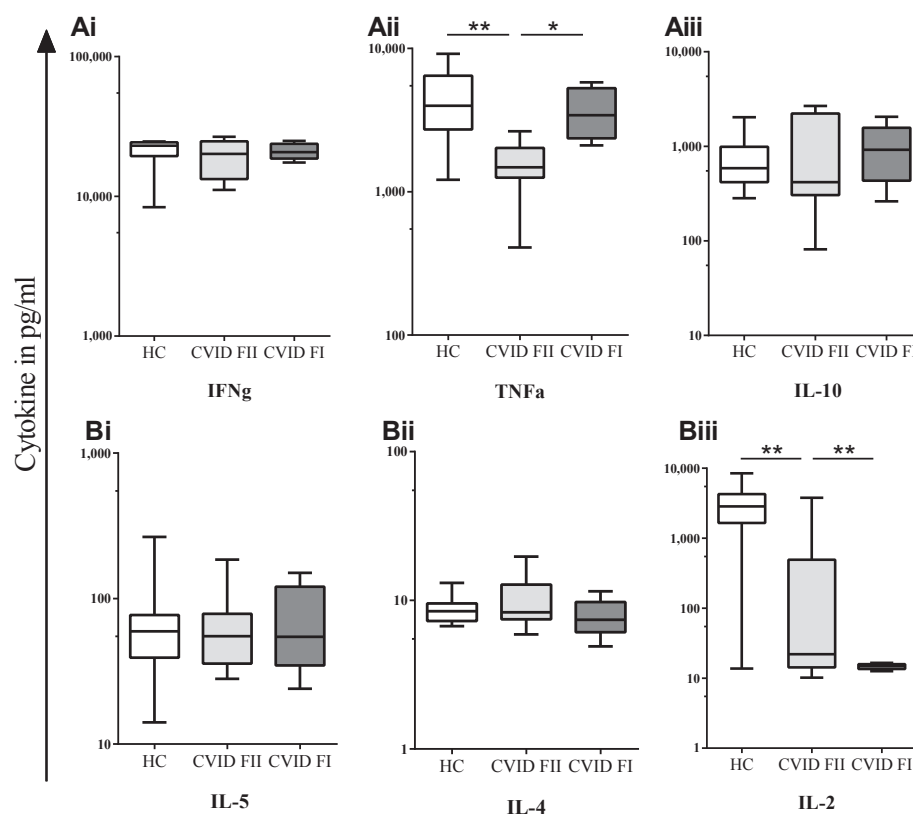


FIG E5. Significant differences in cytokine concentrations of culture supernatants between HC subjects and patients with CVID for TNF- α and IL-2. The x-axis shows the 3 groups: HC subjects and patients with CVID classified as in the FII or FI groups. On the y-axis, cytokine concentrations in picograms per milliliter are depicted for the following T cell-relevant cytokines: IFN- γ (Ai), TNF- α (Aii), IL-10 (Aiii), IL-5 (Bi), IL-4 (Bii), and IL-2 (Biii). Significant differences between the groups were calculated by using the Mann-Whitney *U* test and can only be seen for TNF- α (** P = .0012 and * P = .0166) and IL-2 (** P = .0034 for HC subjects to FII group patients and ** P = .0047 for FII to FI group patients). Freiburg classification: FI group patients have less than 0.4% (triangles) and FII group patients have greater than 0.4% (squares) CD27⁺IgM⁺IgD⁺ memBcs of total lymphocytes.